Inhibition of Cyclooxygenase (COX)-2 Expression by Tet-Inducible COX-2 Antisense cDNA in Hormone-Refractory Prostate Cancer Significantly Slows Tumor Growth and Improves Efficacy of Chemotherapeutic Drugs

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ABSTRACT

Purpose: **Overexpression of the proinflammatory enzyme cyclooxygenase (COX)-2 is associated with the progression of various malignancies; the role of COX-2 in prostate cancer is less clear. The significance of COX-2 in prostate cancer growth and response to chemotherapy was investigated in an androgen-refractory prostate cancer cell line using a Tet-inducible antisense COX-2 expression system.**

Experimental Design: **An antisense COX-2 cDNA construct under the control of a doxycycline-inducible promoter was transfected into a prostate cancer cell line, PC-3ML. Modulations of cell growth, apoptosis, and chemosensitivity in the presence or absence of doxycycline were analyzed. Tumor incidence, growth rate, and response to two cytotoxic drugs, COL-3 [chemically modified tetracycline-3-(6-demethyl-6-deoxy-4-dedimethylamino-tetracycline)] and Taxotere (docetaxel), were investigated in tumor xenografts. Apoptotic incidences and tumor microvessel density in tumors were determined by immunohistochemistry.**

Results: **Conditional suppression of COX-2 in PC-3ML caused reduced cell proliferation, decreased levels of phosphorylated AKT, G_0-G_1 arrest, and increased apoptosis and caspase-3 activity. Suppression of COX-2 increased Bax protein and decreased Bcl-x_L protein *in vitro*. COX-2 antisense-expressing PC-3ML tumors showed a 57% growth delay compared with nontransfected or vector controls. Oral administration of COL-3 (40 mg/kg, oral gavage) or Taxotere (2.3 mg/kg, intraperitoneally; 3× per week) in tumor-bearing mice further slowed tumor growth (65% and ~94%, respectively). Compared with the control group, the occurrence of apoptosis in antisense COX-2 tumors was eight times higher, and the tumor microvessel density was three times lower.**

Conclusions: **These results provide direct evidence that constitutive expression of COX-2 in prostate cancer has both angiogenic and cytoprotective functions. Suppression of tumor cell COX-2 is sufficient to enhance chemotherapy response in prostate cancer.**

INTRODUCTION

Cyclooxygenase (COX)-1 and COX-2 are the two isoforms of COX enzymes that convert arachidonic acid into several eicosanoids, such as prostaglandin (PG), the thromboxins, and prostacyclin, which participate in normal physiology and are implicated in inflammation (1). Whereas COX-1 is constitutively expressed in most tissues, COX-2 is an inducible enzyme stimulated by cytokines, growth factors, oncogenes, or tumor promoters during inflammation or malignancy and causes the release of cytoprotective and proangiogenic cytokines (2, 3).

The role of COX-2 in carcinogenesis has been extensively documented, and in some tumor models, COX-2 overexpression is implicated in metastasis and the development of resistance to antitumor agents (4, 5). However, a clearer understanding of the role of COX-2 in tumor progression is needed in conditions such as prostate and breast cancers, and the potential of COX-2 to protect tumor cells against drug-induced toxicity is unclear. For instance, at present, whether increased expression of COX-2 is associated with prostate cancer (6), in contrast to other cancers, such as colorectal malignancies (7), is unresolved. Whereas some reports suggest that there is no significant increase in COX-2 expression in prostate tumor tissues when compared with normal tissues (8, 9), others have reported a direct association of prostate tumor grade and poorly differentiated prostate cancer with high COX-2 levels (10–12). In addition, significant inhibition of tumor growth by COX-2 inhibitors in prostate cancer models expressing both high and low levels of COX-2 has introduced ambiguity regarding the role of COX-2 in prostate cancer (13). Furthermore, the purported role of COX-2 overexpression in hormone-refractory prostate cancer (HRPC) is far from clear. To illustrate this further, the induction of antiproliferative activity using synthetic inhibitors of COX-2 such as celecoxib (CXB) was initially thought to be caused by the inhibition of COX-2 activity (14), but a series of recent reports seriously dispute that conclusion. These reports suggest that the antitumor effect of COX-2 inhibitors is primarily due to increases in apoptotic activity resulting from exposure to these agents (15). The antitumor effect is most likely independent of COX-2 expression (16). Antitumor drugs induce inflammatory and stress responses in tumor cells, which ultimately protect the

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tumor cells against drug-induced cytotoxicity; it is not clear whether this protection is obligately dependent on COX-2 activity (17, 18). We hypothesized that up-regulation of COX-2 expression is a mechanism by which tumor cells can reduce the cytotoxic effects of anticancer drugs. We further hypothesized that inhibiting the high level expression of COX-2 should increase the efficacy of chemotherapy drugs *in vitro* and *in vivo*.

Most established HRPC cells *in vitro* constitutively express COX-2, whereas androgen-responsive prostate cancer cells (*e.g.,* LNCaP cells) express very little COX-2 activity, despite high mRNA expression (19). Both cell types express low levels of COX-1, a noninducible housekeeping enzyme. The inducible suppression of COX-2 by stable transfection of Tet-inducible antisense COX-2 plasmid offers an unrivaled opportunity to investigate the role of COX-2 in HRPC cells that constitutively express that enzyme by eliminating the fluctuation due to cell line variations. In the present report, we have exploited this technique and demonstrated that suppression of COX-2 expression and consequent suppression of COX-2 activity render the cells significantly less aggressive, increase cytotoxicity, and sensitize the xenografts to antiproliferative and antimetastatic drugs.

MATERIALS AND METHODS

Chemicals and Antibodies. COL-3 [chemically modified tetracycline-3(6-demethyl-6-deoxy-4-dedimethylaminotetracycline)] was from Collagenex, Inc. (Newtown, PA). Docetaxel (Taxotere; Aventis Pharmaceuticals, Collegeville, PA) was obtained from a local pharmacy. A 1,000× stock solution of Taxotere prepared in dimethyl sulfoxide was used *in vitro*, and for *in vivo* studies, Taxotere was prepared in a vehicle (13% EtOH in water with 0.05% Tween 80) for intraperitoneal injection.

Cells and Tumor Lines. PC-3ML, a metastatic variant of the PC-3 cell line (20), was a gift from Dr. Mark E. Stearns (Drexel University, Philadelphia, PA). PC-3ML was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc., Norcross, GA) and gentamicin (10 μg/mL), as described previously (21, 22).

Measurement of Prostaglandin E_2 and Vascular Endothelial Growth Factor by Enzyme-Linked Immunosorbent Assay. PGE_2 and vascular endothelial growth factor (VEGF) released by prostate cancer cells in culture-conditioned medium (CCM) were measured using a competitive immunoassay kit (PGE_2) and an enzyme-linked immunosorbent assay (ELISA) kit (VEGF), respectively (both from R&D Systems, Minneapolis, MN). Briefly, the CCM from prostate cancer cells (5×10^4 cells per well; 12-well plate) cultured with or without drugs for 24 hours, was collected and assayed for PGE_2 or VEGF as per the supplier's instructions. Levels of PGE_2 or VEGF were expressed as picograms produced per 5×10^4 cells.

Generation of Cyclooxygenase-2 Antisense Cell Lines. Antisense COX-2 cDNA construct under the Tet-On–inducible promoter was a gift from Dr. Jason Morrow (Vanderbilt University Medical Center, Nashville, TN). This antisense construct (1.93 kb) contains the entire coding region of human COX-2 cDNA that is cloned into the *Eco*RV/*Xba*I site, in reverse orientation, into the tetracycline response element containing pUHD.2neo plasmid (23). PC-3ML cells, cultured in 60-mm dishes (5×10^5 cells per dish), were transfected with COX-2 antisense cDNA and Tet-On plasmid, which contains the neomycin (Geneticin) resistance gene for eukaryotic selection, using the Effectene Transfection Kit (Qiagen, Valencia, CA). Stable transfectants were clonally selected in 0.2 mg/mL Geneticin (Invitrogen, Gaithersburg, MD). The COX-2 activities in stably transfected clones treated with or without doxycycline (2 μg/mL) were determined by assaying for PGE_2 in the CCM and by immunoblotting for COX-2 protein. Whole-cell lysates were analyzed by Western blotting with a mouse anti–COX-2 antibody (Cayman Chemicals, Ann Arbor, MI) by standard procedures (24). For the experiments with Tet-On–regulated cells, transfected cells were cultured in separate T-75 flasks containing doxycycline (2 μg/mL) in complete medium for 48 to 72 hours for induction of antisense COX-2 cDNA. Tet-inducible suppression of COX-2 activity was confirmed by assaying for PGE_2 before plating cells for each of the experiments; the CCM from each flask was assayed to confirm PGE_2 production in the respective cell cultures treated with doxycycline (+Dc) or without doxycycline (−Dc).

Cytotoxicity Assays. The sensitivity of various prostate cancer cells to antitumor drugs was evaluated by both colorimetric assay [the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; ref.21] and clonogenic survival assay (colony assay). For the MTT assay, cells were cultured in a 48-well plate (1×10^4 cells per well) and incubated with drugs for 24 to 48 hours, as indicated in Results. After appropriate incubation, the fractions of surviving cells were estimated colorimetrically after a 2-hour incubation with MTT. Sensitivity of transfectants to the antitumor drugs, with or without exogenous PGE_2 (10 μmol/L; Cayman Chemicals) in the cell culture medium for 24 hours, was also determined using the MTT assay. For colony assay, prostate cancer cells were cultured at low density in 60-mm dishes in the presence or absence of drugs (Taxotere or COL-3) for 24 hours and allowed to form colonies for 10 days in the absence of the drugs. Cell colonies were fixed, stained with 0.1% crystal violet, and counted manually using a hand-held electronic counter (25).

Estimation of Apoptotic Activity. A colorimetric ELISA-like assay kit was used (Roche, Indianapolis, IN) to compare the levels of apoptotic activity in various cultures. With this kit, we measured the amount of free mono- and oligonucleosomes in cell lysates from cultures undergoing apoptosis.

Determination of Total and Phosphorylated Protein Kinase B (AKT). PC-3ML and 6C without doxycycline (6C−Dc) or with doxycycline (6C+Dc) cells (20,000 cells per well per 0.1 mL) were seeded in a 96-well plate for 24 hours and treated with Taxotere (30 nmol/L) or COL-3 (10 μmol/L) for 24 hours. Total and phosphorylated AKT levels were determined using a colorimetric fast-activated cell-based ELISA kit (Active Motif, Carlsbad, CA).

Determination of Cell Cycle Phase Fractions. PC-3ML, 6C−Dc, and 6C+Dc cells were cultured in 60-mm dishes (1×10^5 cells per dish) for 2 days before preparing them for cell cycle analysis. Cultures were harvested, and the nuclei were labeled with propidium iodide (25 μg/mL) in a single step using a hypotonic citrate buffer containing nonionic detergent Nonidet P-40 (0.04%), as described previously (22). The amount of

propidium iodide bound to cellular DNA was profiled in a Beckman-Coulter EPICS XL flow cytometer, and DNA histogram data were collected in list mode. The cell cycle phase fractions in each sample were estimated using an analysis program (Modfit; Verity Software Inc., Topsham, ME).

Determination of Caspase-3 Activity. The caspase-3 activity in prostate cancer cells was determined using the EnzChek Caspase-3 Activity Assay Kit 2 (Molecular Probes, Eugene, OR). This kit provides the substrate for caspase-3 [Z-DEVD linked with rhodomine 110 (R110), which is cleaved by active caspase-3 present in the cell lysate that releases R110]. The free R110 released is proportional to the active caspase-3 in the cell lysate, which is read in a fluorescence plate reader (excitation and emission wavelengths of 499 and 528 nm, respectively).

Immunoblotting. Cell lysates (~50 μg/lane) prepared from various cultures and tumor explants were analyzed by immunoblotting (Western blotting) by standard procedures as described previously (23). The antibodies used to identify various antigens were as follows: rabbit anti–COX-1 and VEGF IgG (both rabbit IgG; 1 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti–COX-2 IgG (1 μg/mL; Cayman Chemicals); anti–Bcl-2, anti–Bcl-x_L, and anti-Bax antibodies (all mouse IgG; 1 μg/mL; BD Biosciences, Bedford MA); rabbit anti–X chromosome-linked inhibitors of apoptosis protein (XIAP) antibody (0.5 μg/mL; Trevigen, Gaithersburg, MD); and cytokeratin 8/18 (mouse monoclonal IgG; 2.5 μg/mL; Novacastra/Vector Labs, New Castle upon Tyne, United Kingdom). These antibodies were used by standard procedures as described previously (24). Alkaline phosphatase (AP)-conjugated goat antirabbit or goat antimouse antibodies with an AP color detection system (Bio-Rad, Hercules, CA) or appropriate horseradish peroxidase-conjugated secondary antibodies (antirabbit or antimouse IgG; Cayman Chemicals) with enhanced chemiluminescence system (ECL Plus kit; Amersham, Piscataway, NJ) were used to detect the antibody-bound proteins in the blot. The blotted membrane was exposed to X-ray film after enhanced chemiluminescence development to visualize the antigen or developed directly with AP-specific chromogens (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Bio-Rad). The relative band intensities were compared using Kodak 1D-Image Analysis Software (Eastman Kodak, NY). Changes in the protein expression between treated and untreated samples were determined after normalizing the band intensity of each lane to that of cytokeratins.

Direct Quantitation of Cyclooxygenase-2 Messenger RNA. A colorimetric assay for quantifying COX-2–specific mRNA (Quantikine mRNA detection kit; R&D Systems) was used to determine the amount of COX-2 mRNA present in PC-3ML cells. For this assay, the total RNA isolated from treated or untreated cells was hybridized to a biotin-labeled oligonucleotide probe and to digoxigenin-labeled detection probes in a 96-well microplate. The concentration of COX-2 mRNA was estimated by including a mRNA calibrator sample, *i.e.*, glyceraldehyde-3-phosphate dehydrogenase mRNA, as per the manufacturer's instructions.

Tumor Generation and Treatment. PC-3ML, 6C−Dc, or 6C+Dc tumors were generated in 6 to 8-week–old athymic mice (Harlan-Sprague Dawley, Indianapolis, IN). The animals were maintained and cared for according to institutional and National Institutes of Health regulations. Suspensions of PC-3ML or clone 6C−Dc cells were mixed with an equal volume of Matrigel solution (BD Biosciences) and implanted (1×10^6 cells; 0.2 mL) subcutaneously on the dorsal flank. Half of the mice with 6C−Dc cells were provided with doxycycline (2 mg/mL) in drinking water. These animals were considered to harbor 6C+Dc tumor. Mice were further divided into four subgroups of six animals each from the above-mentioned two groups (6C−Dc and 6C+Dc), one with doxycycline in drinking water, and one without. We used an effective dose of COL-3 (40 mg/kg, daily, oral gavage) to reduce tumor growth by half as described elsewhere (21). The Taxotere dose selected was 2.3 mg/kg (intraperitoneally, 3x per week), as described previously (26). Each subgroup of animals was dosed with 0.5 mL of 1% CMC, 0.1 mL of PBS (pH 7.4; intraperitoneally, 3× per week), COL-3 (40 mg/kg, oral gavage, in 0.5 mL of 1% CMC), or Taxotere (2.3 mg/kg body weight; intraperitoneally, 3× week) for 42 days. After the tumors became palpable (~day 7), tumor volumes (TVs) were measured three times a week with a vernier caliper, and the volume was approximated to an ellipsoid (length × height × width × 0.524). Mice were euthanized 42 days after tumor implantation, by which time the TVs in the control group had reached between ~800 and 1,000 mm^3. At euthanasia, tumors were excised and weighed. Primary tumor cell cultures were set up from at least three tumors from each group; the remainder was processed for immunohistochemistry as described below.

Estimation of Microvessel Density (CD34+ Cells) by Immunohistochemistry. Paraffin-embedded 5-μm tissue sections were placed on positively charged slides. Sections were dewaxed, rehydrated, and steamed in an antigen retrieval solution [buffered citrate (pH 6.0)] in a 90°C water bath. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol, and nonspecific binding sites were blocked with 10% preimmune rabbit serum. Sections were incubated for 18 hours at 4°C with a rat antimouse CD34 monoclonal antibody (2.5 μg/mL; PharMingen/BD Biosystems), washed, incubated with a streptavidin-peroxidase–based detection reagent set containing biotin-labeled rabbit antirat IgG (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA), and developed with diaminobenzidine for 7 minutes. The slides were counterstained with hematoxylin, and the microvessel density (MVD) was determined by counting the anti-CD34–stained microvessels, as described previously (27). Briefly, the area with the highest MVD in each tissue specimen, identified using a ×4 objective, was designated as a "hot spot." The microvessels in the hot spots were counted under the ×10 objective (×100 magnification). A single, countable microvessel was defined as any vessel with lumen and endothelial cell or endothelial cell cluster stained positively for CD34 antibody. The MVD from each slide was determined in three separate fields per hot spot. The MVD was expressed as the mean ± SD for each subgroup, based on observations by two independent readers who selected the hot spots and counted the microvessels.

***In situ* Detection of Apoptosis in Tumor Tissue by Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay.** Apoptotic cells in the tumor tissues were detected in fresh frozen sections by the terminal deoxynucleo-

tidyl transferase-mediated nick end labeling (TUNEL) method, using the *In situ* Cell Death Detection Kit POD (Roche). Briefly, 8-μm tumor tissue sections embedded in OCT were air dried and fixed with a 4% paraformaldehyde solution and processed as per the instructions provided in the kit. Tissue sections were developed with diaminobenzidine, counterstained with hematoxylin, dehydrated, and mounted. Specificity of the nuclear staining pattern from at least three different fields was assessed by three separate readers observing the slides under a ×20 objective (×200 magnification).

Statistical Analysis. Triplicate samples were assayed in all *in vitro* experiments in three independent assays. The significance of the observations was tested by analysis of variance using the Instat Statistical Program (Graph Pad Software Inc., San Diego, CA). Either the Tukey-Kramer multiple comparisons test or Bonferrroni's test was used to test the significance of the tumor growth differences between the various treatment groups. Other data were analyzed using Student's *t* test or the Mann-Whitney rank-sum test, as appropriate (see Results).

RESULTS

Cytotoxic Chemodrugs Increase Cyclooxygenase-2 Messenger RNA Expression and Prostaglandin E_2 Release in PC-3ML Cells. Treatment of PC-3ML cultures with COL-3 or Taxotere for 24 hours resulted in a significant increase (42 ± 2.1% and 44.5 ± 1.3%, respectively) in PGE_2 release as compared with that of untreated control (Fig. 1*A*). To ascertain that the increase in PGE_2 levels was primarily due to an increase in COX-2 expression, we measured the COX-2 mRNA in PC-3ML cells treated with either COL-3 (10 μmol/L) or Taxotere (30 nmol/L) for 24 hours. The levels of COX-2 mRNA were significantly increased (6× and 11×, respectively) in drug-treated cells, as compared with the levels in untreated cells (Fig. 1*B*).

Previous reports from our laboratory and others (28, 29) have shown that HRPC cells such as PC-3 or PC-3ML have significantly reduced chemosensitivity to the drugs Taxotere and COL-3 as compared with other, less aggressive prostate cancer cells. One of the causes of this reduced chemosensitivity could be high COX-2 activity resulting in increased PGE_2 production after chemodrug treatment. Therefore, we examined whether an alteration in COX-2 expression results in an alteration in drug sensitivity. For this, we chose to conditionally down-regulate COX-2 expression in PC-3ML cells using the antisense COX-2/Tet-On expression system.

Selection of PC-3ML Clones with Tet-Inducible (+Dc) Antisense Cyclooxygenase-2 Expression. We characterized seven clones, and we selected clone 6C, which, under −doxycycline conditions, produced a normal amount of PGE_2 (1,077 ± 72.5 pg of PGE_2 per 5×10^4 cells, compared with 1,030 ± 147.2 pg of PGE_2 per 5×10^4 PC-3ML cells) but produced significantly less PGE_2 (200 ± 18.5 pg of PGE_2 per 5×10^4 cells; an 80% reduction) in the presence of doxycycline (Tet-On). The level of PGE_2 synthesis was unchanged (Fig. 1*C*) when clone 6C+Dc cells were treated with either COL-3 (10 μmol/L) or Taxotere (30 nmol/L) for 24 hours, indicating that the increase in PGE_2, wild-type PC-3ML, or 6C−Dc after cytotoxic drug treatment is likely due to an elevated COX-2 activity and not COX-1 activity. Conversely, the low level of PGE_2 in clone 6C+Dc was due to COX-1 activity, which was abolished by treatment with the COX-1–specific inhibitor SC 560 (Calbiochem, La Jolla, CA; data not shown). COX-2 immunoblot showed a >90% decrease in the level of COX-2 in doxycycline-treated samples, as compared with that in non–doxycycline-treated samples (Fig. 1*C*, *inset*). Furthermore, treatment of clone 6C cells (6C−Dc *versus* 6C+Dc) with Taxotere 1 or COL-3 showed a significant increase in the expression of COX-2 in the 6C−Dc samples, as determined by immunoblotting (~50% increase in the band intensity). No such increase was detected in the cells treated with doxycycline. Little induction of COX-2 is seen in cells treated with doxycycline (Fig. 1*C*, *inset*, *Lanes 2* and *3 versus Lanes 5* and *6*, respectively).

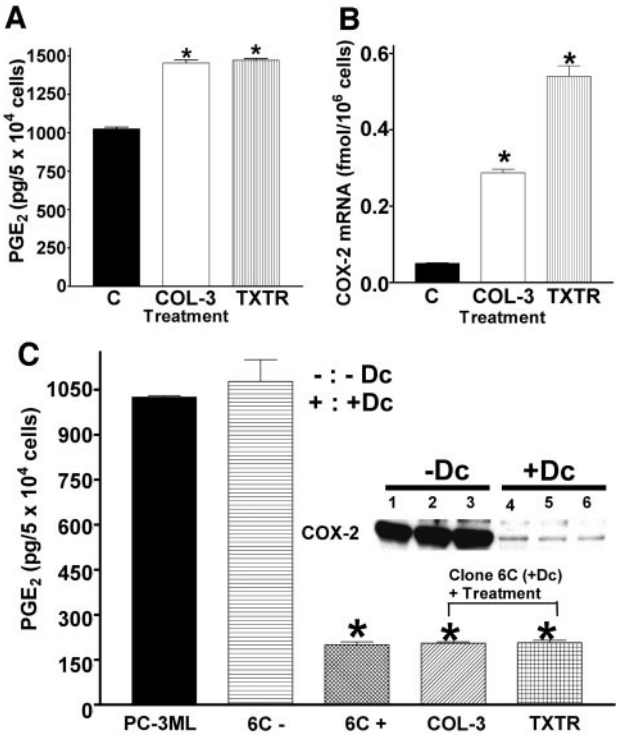


Fig. 1 *A* and *B*, increased PGE_2 release (*A*) and COX-2 mRNA expression (*B*) in PC-3ML cells. *A*. PGE_2 levels were measured in CCM by ELISA after treatment with COL-3 (10 μmol/L) or Taxotere (30 nmol/L; *TXTR*) for 24 hours. *B*, COX-2 mRNA in untreated and COL-3 (10 μmol/L)- or Taxotere (30 nmol/L)-treated PC-3ML cells (24 hours). Data presented are from three independent experiments. *C*, characterization of antisense COX-2 clones of PC-3ML. Tet-inducible antisense clones of PC-3ML cells were cultured with (+) or without (−) doxycycline (2 μg/mL; *Dc*) for 48 hours. PGE_2 levels in CCM were compared with PGE_2 levels of parental PC-3ML cells. Data represent mean ± SE ($n = 3$; *, $P < 0.05$, Student's *t* test, PC-3ML *versus* transfectants). *Inset*, immunoblot of COX-2 protein in cell lysates of clone 6C−Dc [untreated, Taxotere treated, or COL-3 treated (*Lanes 1–3*, respectively)] or clone 6C+Dc [(untreated, Taxotere treated, or COL-3 treated (*Lanes 4–6*, respectively)]. Equal loading of proteins in each lane was ensured by determining cytokeratin bands in the same blot (data not shown).

Antisense Down-Regulation of Cyclooxygenase-2 Causes Cell Cycle Arrest (G_0-G_1 Arrest). It was reported that COX-2 inhibition by CXB causes cell cycle arrest at G_0-G_1

(15, 30). The percentage of cells in the G_0-G_1 phase of the cell cycle was significantly greater (44 ± 1.6%) in 6C+Dc cells than in 6C−Dc cells ($P < 0.05$). In addition, as summarized in Table 1, we observed a significant decrease (47 ± 0.84%) in the S-phase fraction of antisense COX-2 6C+Dc cells as compared with 6C−Dc or PC-3ML cells ($P < 0.05$).

Increased Drug Sensitivity of Antisense Cyclooxygenase-2 (6C+Dc) Cells. As shown in Fig. 2*A*, the clonogenic survival of 6C+Dc without treatment with cytotoxic drug (untreated control) was itself significantly decreased (by 29 ± 12%) when compared with clone 6C−Dc. Furthermore, treatment with COL-3 or Taxotere caused a further decrease in clonogenic survival by 72 ± 1.3% and 83 ± 1.42%, respectively, in survival of clone 6C+Dc as compared with untreated 6C−Dc. For instance, the clonogenic survival of 6C−Dc cells treated with Taxotere (30 nmol/L, 24 hours) was 38 ± 4.2% of untreated 6C−Dc cells, whereas the survival of 6C+Dc cells was 31 ± 2.2% of untreated 6C+Dc cells. Clonogenic survival of 6C−Dc cells treated with COL-3 was 56 ± 2.7% of untreated 6C−Dc cells, and for 6C+Dc, the reduction was 44 ± 2.4% of untreated 6C+Dc cells.

Increased Apoptosis in Antisense Cyclooxygenase-2 (6C+Dc) Cells. Data from Cell death ELISA showed an approximately 2-fold increase in basal apoptotic levels in 6C+Dc cultures compared with that of 6C−Dc cultures (Fig. 2*B*). This increase in apoptotic activity was statistically significant (*t* test, $P < 0.05$). In addition, apoptosis was significantly increased in both cultures (6C−Dc and 6C+Dc) incubated with Taxotere (30 nmol/L) or COL-3 (10 μmol/L) for 24 hours. The increase in soluble nucleosome levels in the cell lysates of Taxotere-treated cultures in the 6C+Dc group was ~3-fold as compared with the untreated 6C+Dc cell lysates but was 4.5-fold when compared with the 6C−Dc untreated group. Apoptosis was also increased in 6C−Dc cultures by 3.5-fold in the Taxotere-treated group, as compared with the untreated 6C−Dc group. A 2-fold increase in apoptotic activity in both groups (6C−Dc and 6C+Dc) was also observed in 6C+Dc cultures treated with COL-3 (Fig. 2*B*).

AKT Down-Regulation Decreased Bcl-x_L and Increased Bax Expression in Antisense Cyclooxygenase-2 Cells. As shown in Fig. 3*A*, we observed a consistent *increase* in the levels of phosphorylated AKT after COL-3 (15%) or Taxotere (20%) treatment in 6C−Dc cells as compared with the untreated controls. However, there was a significant *decrease* (50 ± 0.56%) in the levels of phosphorylated AKT in COX-2–depleted 6C+Dc cells as compared with the 6C−Dc cells. The levels of phosphorylated AKT in COL-3–treated and Taxotere-treated clone 6C cultures (with or without doxycycline) showed a modest increase.

Table 1 Effect of COX-2 expression on cell cycle phase fractions

Phase	PC-3ML	Clone 6C−Dc	Clone 6C+Dc
G_0-G_1	47.83 ± 3.05	48.91 ± 3.30	68.89 ± 2.30
S	32.06 ± 5.25	31.67 ± 2.80	16.80 ± 4.30
G_2-M	19.96 ± 4.02	19.42 ± 4.10	16.80 ± 3.80

NOTE. Data are from three separate determinations and represent the mean ± SE.

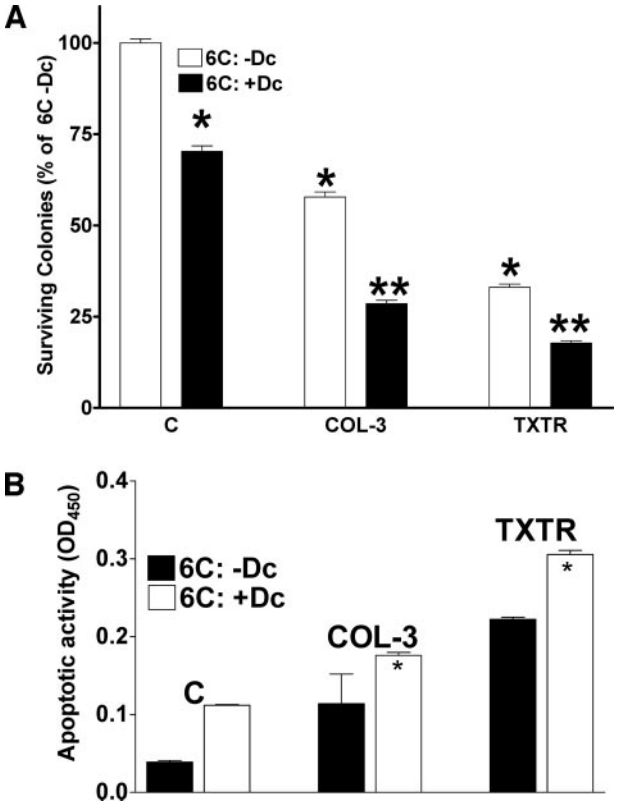
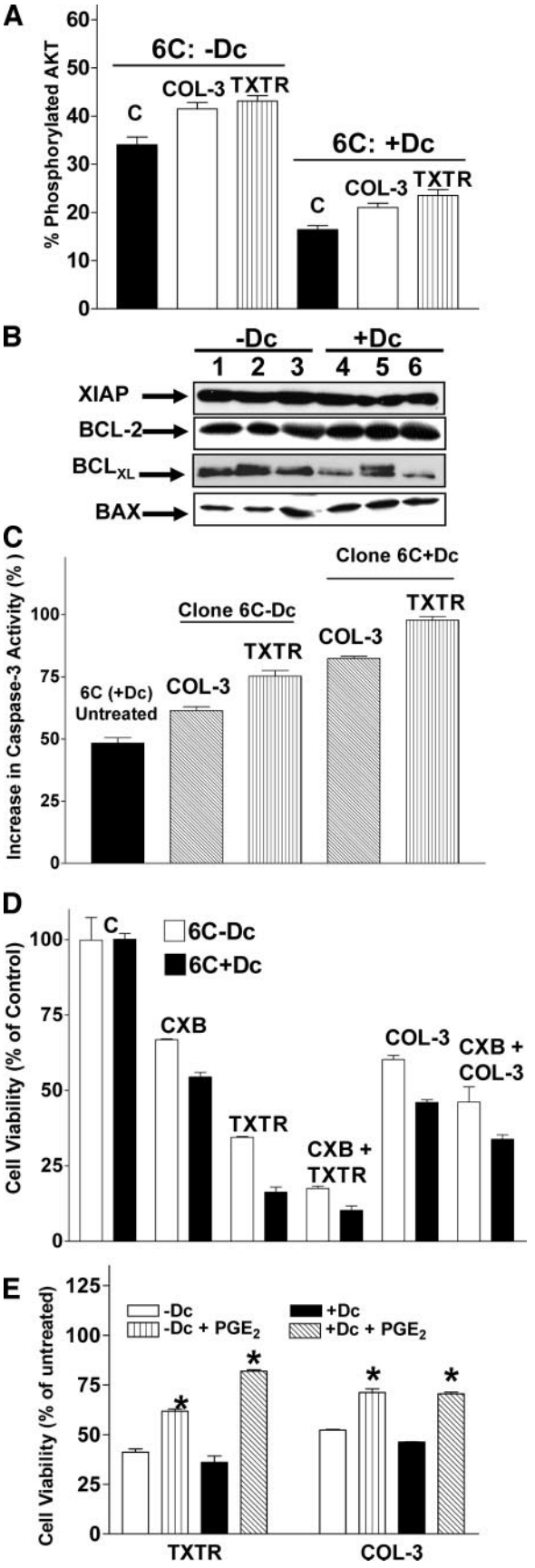


Fig. 2 *A*, clonogenic survival of clone 6C−Dc or 6C+Dc cells with or without chemodrugs. 6C−Dc or 6C+Dc cells were cultured in 60-mm culture dishes (~1,000 cells per dish) and exposed to COL-3 (10 μmol/L) or Taxotere (30 μmol/L; *TXTR*) for 24 hours. Data represent mean ± SE from three assays. *, $P < 0.05$, *t* test, 6C+Dc *versus* 6−Dc. *B*, determination of apoptotic activity in 6C−Dc or 6C+Dc. Apoptotic activities in 6C−Dc or 6C+Dc cells (1×10^4 cells per well; 48-well plates) treated with COL-3 (10 μmol/L) or Taxotere (30 nmol/L) for 24 hours were assayed. Data shown are mean ± SE ($n = 3$). *, $P < 0.05$, *t* test, 6C−Dc *versus* 6C+Dc for each treatment. **, $P < 0.05$, *t* test, 6C−Dc *versus* 6C+Dc for each treatment.

Western blot analysis of protein levels of the antiapoptotic proteins Bcl-2 and Bcl-x_L revealed differences in their expression levels (Fig. 3*B*). Bcl-2 expression showed no significant difference between the Taxotere-treated or COL-3–treated 6C−Dc and 6C+Dc cells. On the other hand, however, the level of bcl-x_L was significantly down-regulated in 6C+Dc cells as compared with 6C−Dc cells under the same conditions. In addition, bcl-x_L levels were elevated in Taxotere-treated cells in 6C−Dc and 6C+Dc clones compared with the untreated cells. The level of Bax, a proapoptotic protein, was significantly increased in COX-2–depleted clone 6C+Dc (whether treated or untreated) when compared with the COX-2–expressing 6C−Dc cells. We next investigated whether COX-2 down-modulation affects inhibitors of apoptosis proteins, especially the dominant inhibitors of apoptosis protein, XIAP (31). Western blot analysis showed no significant change in the protein levels of XIAP, regardless of treatment or COX-2 expression status (Fig. 3*B*).

Increase in Caspase-3 Activity in Cells Expressing Cyclooxygenase-2 Antisense Complementary DNA. As shown in Fig. 3*C*, we observed significantly increased caspase activity



in clone 6C+Dc cells when compared with clone 6C−Dc cells. The caspase activity was increased in untreated clone 6C+Dc cells by 48 ±1.3%. The increased caspase activity was further enhanced by exposure to COL-3 or Taxotere by 82 ± 2.5% and 94 ± 2.4%, respectively, in 6C+Dc compared with untreated 6C−Dc (control).

Effect of Cyclooxygenase-2 Inhibitor Celecoxib or of Prostaglandin E_2 with or without Chemodrugs on 6C−Dc and 6C+Dc Cells. As shown in Fig. 3*D*, the COX-2 inhibitor CXB had significant cytotoxicity in both 6C−Dc and 6C+Dc cultures. Specifically, the cytotoxicity of CXB at 10 μmol/L was independent of COX-2 expression, indicating that CXB exerts cytotoxicity in these cells, independent of its COX-2 inhibitory activity, an observation reported previously by Hsu *et al.* (14). Furthermore, combined addition of CXB and Taxotere significantly increased cytotoxicity in both transfectants. Cytotoxicity resulting from combined treatment with COL-3 (10 μmol/L) and CXB (10 μmol/L) was not significantly greater than that due to either agent alone ($P = 0.15$).

The drug sensitivity of clone 6C−Dc and 6C+Dc cells was decreased in those cells exposed to chemodrugs in the presence of exogenous PGE_2 (10 μmol/L) for 24 hours, as compared with those in the absence of PGE_2 (Fig. 3*E*). The sensitivity of the 6C−Dc cells to Taxotere decreased by 33 ± 1.2%, and the drug sensitivity toward COL-3 decreased by 27 ± 0.09%. Similarly, 6C−Dc cells showed a 55 ± 2.1% decrease in sensitivity to Taxotere and a 35 ± 1.9% decrease (*i.e.*, increase in viability) after exposure to COL-3. These results provide evidence that the observed increase in drug sensitivity in 6C+Dc cells is due to the absence of COX-2/PGE_2 in these cells.

Growth of Clone 6C−Dc and 6C+Dc Tumors in Athymic Mice. Tumors were incident in all groups of animals, regardless of antisense COX-2 induction. However, there was a significant delay (25%; $P < 0.05$) in the appearance of palpable

Fig. 3 Percentage of phosphorylated AKT in clone 6C+Dc (*A*), and immunoblot of pro- and antiapoptotic proteins (*B*). *A*, the ratio of phosphorylated AKT to total AKT (expressed as a percentage) in clone 6C−Dc or 6C+Dc cells treated with COL-3 (10 μmol/L) or Taxotere (30 μmol/L; *TXTR*) was determined by ELISAs. *B*. Protein levels of XIAP Bcl-2/Bcl-x_L and Bax were determined in clone 6C−Dc and clone 6C+Dc with or without Taxotere (30 μmol/L; *Lanes 2* and *5*) or COL-3 (10 μmol/L; *Lanes 3* and *6*) by Western blot analysis. Differences in the percentage of phosphorylated AKT were significantly less in 6C+Dc cells than in the 6C−Dc cells, all groups ($P < 0.05$ compared with respective untreated control, *t* test). C. Caspase-3 activity in clone 6C−Dc or 6C+Dc. Clone 6C cells (± doxycycline) were treated with COL-3 (10 μmol/L) or Taxotere (30 μmol/L) for 24 hours. Increase in caspase-3 activity (%) = [(activity in treated cell lysate − activity in untreated cell lysate) × 100 ÷ (activity in untreated 6C−Dc cell lysate)]. *D*, effect of CXB on viability of clone 6C−Dc or 6C+Dc cells with or without chemodrugs. Clone 6C−Dc or 6C+Dc was exposed to CXB (10 μmol/L), COL-3 (10 μmol/L), Taxotere (30 μmol/L), CXB + COL-3, or CXB + Taxotere for 24 hours. Cell viability after treatment was determined using the MTT assay. *E*, effect of PGE_2 on viability of clone 6C−Dc or 6C+Dc cells with or without chemodrugs. Clone 6C−Dc or 6C+Dc cells were exposed to CXB (10 μmol/L), COL-3 (10 μmol/L), Taxotere (30 μmol/L), CXB + COL-3, or CXB + Taxotere with or without 10 μmol/L PGE_2 for 24 hours. Cell viability was determined using the MTT assay. *, $P < 0.05$, all pairs, Tukey-Kramer test.

tumors in the clone 6C+Dc groups. The mean TV in the untreated 6C+Dc group was significantly less (57 ± 1.62%) than that of either the clone 6C−Dc or PC-3ML control groups. In Taxotere treatment groups, there were significant reductions in mean TV of clone 6C−Dc (80 ± 1.63%) and clone 6C+Dc (85%) as compared with their respective untreated controls. When the Taxotere groups were compared with the untreated parent 6C−Dc cells and/or PC-3ML cells, the reduction in TV increased to 94%. In addition, tumors in the 6C+Dc group treated with Taxotere showed very little growth starting from day 25 after tumor implant, resulting a mean volume of < 50 mm^3 on day 42. Overall, the most effective combination for tumor growth control was antisense COX-2 + Taxotere.

A significant decrease in tumor growth was also observed in mice treated with COL-3. On day 42, the decrease in mean TVs for the COL-3–treated 6C−Dc and 6C+Dc groups were 36 ± 1.35% and 21 ± 3.6%, respectively. Although, COL-3 treatment of 6C−Dc tumors reduced the mean TV by 65% compared with the 6C−Dc vehicle-only control ($P < 0.004$), the COL-3–treated 6C +Dc group did not demonstrate significant growth delay (Mann-Whitney test, $P = 0.17$).

As shown in Fig. 4*B*, quantitative analysis of tumor weight measured on day 42, at the time of necropsy, corroborated the findings based on terminal TVs. Tumor weights in the 6C−Dc group treated with COL-3 and Taxotere were 36 ± 0.53% and 48.0 ± 1.2% lower, respectively, as compared with the untreated (vehicle only) 6C−Dc group. Compared with the untreated 6C−Dc group, tumor weights in the COL-3/Taxotere-treated 6C−Dc groups decreased 55 ± 1.2% and 87 ± 0.93%, respectively. Treatment had minimal adverse effect on the body weight and health of the animals. Regardless of treatment, body weight of animals increased about 5% to 20% during the 6 weeks of treatment.

Increased Apoptosis and Reduced Microvessel Density in the Antisense Cyclooxygenase-2 Tumors. As shown in Fig. 5*B*−*D*, the number of apoptotic cells in tumor sections was significantly higher in antisense COX-2 6C+Dc, as compared with that in 6C−Dc (Fig. 5*A*). The incidence of apoptotic cells was at least 3-fold higher in untreated 6C+Dc tumors than in the untreated 6C−Dc tumors. In addition, among the Taxotere-treated or COL-3–treated tumors, the difference in apoptotic cell number was 8-fold between 6C+Dc + Taxotere and 6C−Dc + Taxotere, and the apoptotic cell number was 4-fold higher in tumors in the 6C+Dc + COL-3 group than in the 6C−Dc + COL-3 group ($P < 0.05$, Mann-Whitney test; micrograph not shown). We observed an 11-fold increase in apoptotic cells in 6C+Dc + Taxotere tumors compared with that of untreated 6C−Dc.

Decreased occurrence of MVD was clearly visible in tumor sections from the 6C+Dc group, in both Taxotere-treated and untreated (control) tumors (Fig. 5*E*−*H*). The mean MVD among the different treatment groups was also decreased compared with the untreated cohorts. The decrease in MVD counts was 74 ± 1.63%, 62 ± 1.33%, and 39 ± 1.84% in the 6C+Dc + Taxotere, 6C+Dc untreated, and 6C−Dc + Taxotere groups, respectively, as compared with the MVD of the untreated 6C−Dc group, suggesting an association between an increase in apoptosis and a decrease in MVD ($P < 0.04$, Tukey-Kramer test).

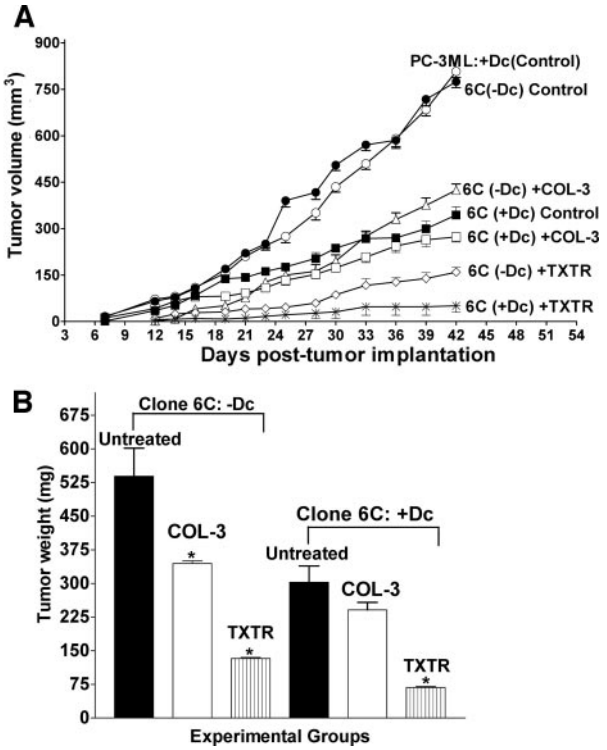


Fig. 4 *A*, tumor growth in clone 6C−Dc or 6C+Dc xenograft-bearing mice treated with Taxotere or COL-3. Mean TVs were significantly low in clone 6C+Dc groups (untreated, COL-3 treated, or Taxotere treated). *B*, mean tumor weights of tumor explants from various experimental groups. Forty two days after tumor cell implantation, all surviving animals were euthanized, and tumor weights were recorded. Mean tumor weights were significantly decreased in the clone 6C+Dc groups (untreated, COL-3 treated, or Taxotere treated) when compared with the clone 6C−Dc group. *, $P < 0.05$, Tukey-Kramer test, treatment (all) *versus* 6C−Dc control.

Secretion of Vascular Endothelial Growth Factor by Tumor Cells in Explant Cultures. The culture supernatant from tumor explant cultures had a significant amount of VEGF (Fig. 6*A*). Culture supernatant of 6C+Dc tumor explants contained a significant decrease in VEGF levels compared with that of 6C−Dc tumors. A further decrease in VEGF secretion was observed in the CCM of 6C+Dc tumors treated with Taxotere as compared with that in the CCM of 6C−Dc tumors (Fig. 6*A*). Although the secretion of VEGF in cultures derived from Taxotere-treated 6C−Dc tumors was significantly less than that of untreated 6C−Dc tumor CCM, it was still higher than that of Taxotere-treated 6C+Dc tumors (35 ± 1.3% and 44 ± 1.5%, respectively; $P \leq 0.05$). The difference between the VEGF level in the CCM of 6C+Dc and 6C+Dc treated with Taxotere was not significant.

Expression of Cyclooxygenase-1 and -2 and Vascular Endothelial Growth Factor Proteins in Cells Derived from Tumors. Western blot analysis of VEGF expression in tumor explant from clone 6C+Dc confirmed the VEGF ELISA results (Fig. 6*B*). We noted a decrease in VEGF protein level (>90%) in clone 6C+Dc and clone 6C+Dc + Taxotere tumor explant cultures as compared with untreated clone 6C−Dc. Further-

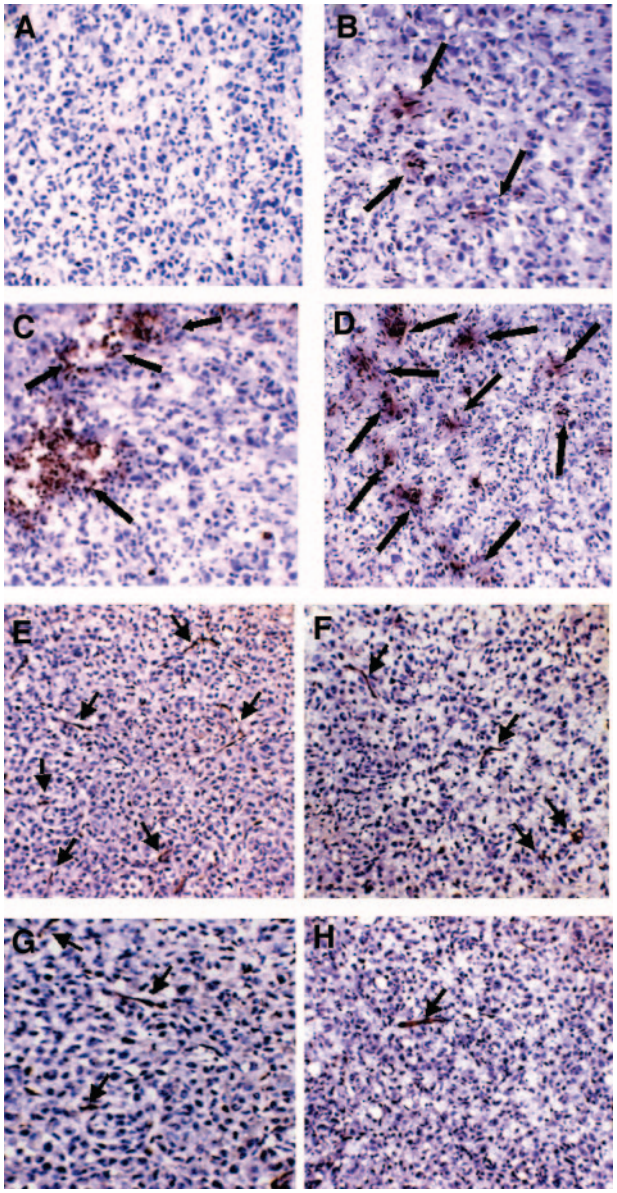


Fig. 5 Photomicrographs showing apoptotic cells in tumor tissue (*A–D*, ×100 magnification), and neovascularization in tumor sections (*E–H*, ×100 magnification). Apoptotic cells in tumor sections (*A*) clone 6C−Dc (untreated), (C), and clone 6C−Dc treated with (Taxotere; 2.3 mg/kg, intraperitoneally, 3× per week). *B*, untreated clone 6C+Dc group; *D*, clone 6C+Dc + Taxotere group. CD34 staining for estimating MVD in tumor sections from (*E*) untreated clone 6C−Dc; (*F*) untreated clone 6C+Dc; (*G*) clone 6C−Dc + Taxotere, and (*H*) clone 6C+Dc + Taxotere.

more, as shown in Fig. 6*B*, protein levels of COX-2 were also decreased (>90%) in clone 6C+Dc untreated and Taxotere-treated tumor explants. Although cultures from all explants expressed COX-1, we did not observe any change (*e.g.,* an increase) in COX-1 expression in tumor explants of 6C+Dc. As showed in Fig. 6*B*, levels of COX-1 were unchanged among 6C−Dc and 6C+Dc tumor explants. In particular, we did not observe any compensatory mechanism for PGE_2 release in the absence of COX-2.

DISCUSSION

Targeting of the inflammatory and cell proliferation pathways in cancer treatment is gaining momentum, largely in response to the observation of the chemopreventive action of nonsteroidal anti-inflammatory drugs in colon cancer (32). Combining cytotoxic drug treatment with anti-inflammatory drug treatment is an attractive alternative to improve the efficacy of either drug because the mechanisms of action of the two types of drugs are likely to be either independent or complementary (33, 34). This concept of combining anti-inflammatory and cytotoxic drugs is based on the preclinical finding that an effective COX-2 inhibitor such as CXB and a cytotoxic (antiproliferative) drug such as Taxotere could be a potent combination therapy for treating aggressive cancers. Several clinical trials are under way in lung, liver, and prostate cancers that use the combination treatment. The results of these studies regarding the efficacy of this treatment modality are not out at this time. A potential problem in the interpretation of the efficacy of this combined treatment modality, if any, could be the ambivalence regarding the mechanism of action of COX-2 inhibitors such as CXB. For instance, a demonstration of the antiproliferative activity of COX-2 inhibitors, even in tumor cell lines that are unable to express detectable levels of COX-2 activity, has led to widespread doubt about the direct involvement of COX-2 in

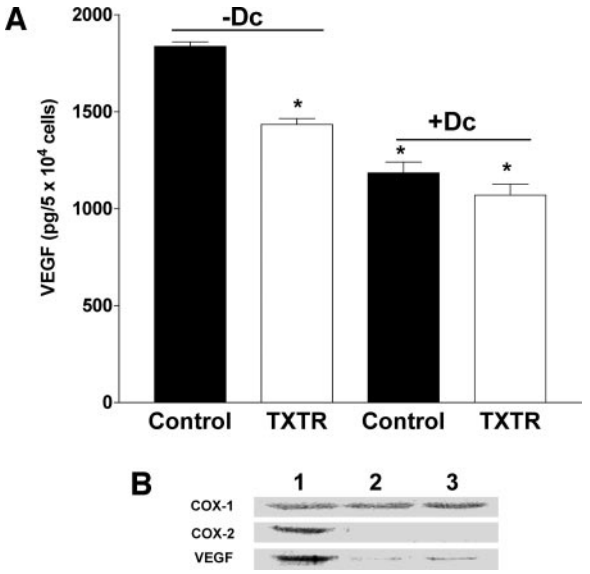


Fig. 6 *A* and *B*, VEGF levels in clone 6C−Dc or 6C+Dc *ex vivo* cultures (*A*) and COX-1, COX-2, and VEGF protein expression in tumor explant cultures (*B*). *A*. VEGF levels were determined in 24-hour CCM from cultures of clone 6C−Dc, clone 6C−Dc + Taxotere (30 μmol/L), clone 6C+Dc, or clone 6C+Dc + Taxotere (30 μmol/L). *, $P < 0.05$, clone 6C−Dc control *versus* all other groups compared pairwise (Mann-Whitney test). *B*, immunoblots of protein levels of COX-1, COX-2, and VEGF in tumor explant cultures from clone 6C−Dc (*Lane 1*), clone 6C+Dc (*Lane 2*), or clone 6C+Dc + Taxotere (*Lane 3*) group of mice using the AP-conjugated color detection system as described in Materials and Methods.

modulating chemotherapy response (35, 36). This situation is further compounded in prostate cancer due to the conflicting observation of COX-2 expression in HRPC (6, 10–12, 37). There are also reports of an apparent disconnect between the potency of COX-2 inhibitors on tumor cell cultures (IC_{50} >10 μmol/L) *versus* that observed to inhibit tumor growth *in vivo* (<1 μmol/L; ref. 38).

An objective of the present work was to attempt to address this ambiguity of the involvement of COX-2 in prostate cancer. Using Tet-On–inducible COX-2 antisense clones of a HRPC model, we demonstrate a direct involvement of COX-2 in prostate cancer cell growth and interaction with cytotoxic drugs. Suppression of tumor cell-derived COX-2 not only decreases the tumor growth rate by increasing apoptosis but also increases the chemosensitivity of HRPC cells to the anticancer drugs used in the study. Subbaramaiah *et al.* (39) showed that taxanes such as Taxotere increase COX-2 transcription and increase the stability of COX-2 transcripts. Interestingly, although we found an ~11-fold increase in COX-2–specific mRNA, the increase in the COX-2 protein or the enzyme product, PGE_2, was modest (50% and 42%, respectively, when we treated cells with the Taxotere IC_{50} dose of 30 nmol/L). This contrasts markedly with the high dose of 10 μmol/L Taxotere used by the earlier investigators. Whether the high levels of COX-2 mRNA, resulting from increased transcription and stability, are offset by increased protein degradation in drug-treated cells is not clear at present. Furthermore, the apparent discrepancy in the overstimulation of COX-2 mRNA but modest increase in COX-2 activity may also be specific to the cell line we investigated. However, a suppression of even this modest increase in COX-2 activity was sufficient to sensitize the tumor cells to increased antitumor activity of Taxotere *in vitro* and *in vivo*.

Our data elucidate the importance of PGE_2 in cell growth and sensitivity toward chemodrugs in highly aggressive HRPC cells, such as PC-3ML. Cell viability and clonogenic survival of COX-2–depleted PC-3ML cells (6C+Dc) were reduced by ~40% without any cytotoxic drugs, and the drug sensitivity increased as compared with 6C−Dc cells (Fig. 3*B*). The decrease in cell viability and clonal survival in COX-2–depleted prostate cancer cells likely contributed to the increase in apoptosis and accumulation in the G_0-G_1 cell cycle compartment in untreated clone 6C+Dc (Table 1). A further increase in drug sensitivity and clonogenic survival in cytotoxic drug-treated clone 6C:Tet-On cells is likely due to similar mechanisms (Fig. 3*B*). Addition of exogenous PGE_2 in COX-2–depleted cells caused a decrease in drug sensitivity (Fig. 3*E*). Interestingly, addition of PGE_2 to 6C−Dc also caused some decrease in sensitivity to chemodrugs; however, the effect of the exogenous PGE_2 addition was more enhanced in COX-2–depleted cells, in which the external PGE_2 caused significantly increased cytoprotection. As observed by others (14), CXB-induced cytotoxicity, independent of its COX-2 inhibitory activity, was also observed in the present study (Fig. 3*D*).

Results presented in this investigation clearly establish that the suppression of COX-2 in tumor cells that constitutively express high levels of COX-2 leads to chemosensitization by changing the ratio of cellular proapoptotic to antiapoptotic proteins. In this respect, we observed a significant reduction in AKT phosphorylation [a component of the antiapoptotic pathway (40)] in COX-2–depleted cells as compared with clone 6C−Dc. Moreover, the protein levels of XIAP, the major inhibitor of apoptosis regulated by AKT (41), remained unchanged, irrespective of the COX-2 status or the drug treatment used in clone 6C cells. It is possible that decreased levels of phosphorylated AKT were not sufficient to induce XIAP-mediated caspase inhibition in COX-2–depleted clones.

The protein levels of pro- and antiapoptotic proteins Bcl-2/Bcl-x_L and Bax also showed distinct alterations. Although the levels of the prosurvival protein Bcl-2 remained unchanged in clone 6C−Dc or 6C+Dc cell cultures, it did not affect the increased caspase-3 activation in clone 6C+Dc. Moreover, we observed a significant decrease in another prosurvival protein, Bcl-x_L, and an increase in the proapoptotic protein Bax in COX-2–depleted cells (Fig. 3*B*). Lebedeva *et al.* (42) have reported that in p53-deficient cells such as PC-3, Bcl-2 plays a limited role, but Bcl-x_L is the major player in opposing drug-induced apoptosis. Furthermore, the increase in Bax in COX-2–depleted cells and enhanced Bax expression in drug-treated, COX-2–depleted cells are likely to be responsible for increased drug-induced apoptosis and caspase-3 activation as reported by Zhang *et al.* (43). Together, these findings suggest that the increased apoptosis in COX-2–depleted cells is possibly due to alteration of the Bcl-x_L to Bax ratio, but not the Bcl-2 to Bax ratio.

The results from xenograft studies showed that suppression of tumor cell COX-2 expression significantly slows tumor growth, regardless of host (stromal COX-2) activity (Fig. 6*A*). This is similar to the COX-2–null mouse and colon cancer model reported by Williams *et al.* (44). It was reported that host COX-2 activity (stromal COX-2 activity) has an insignificant role in tumor growth. Similarly, the results presented in this report clearly indicate that treating COX-2–depleted tumor xenograft-bearing mice with a significantly low dose of the anticancer drug Taxotere (2.3 mg/kg, intraperitoneally, 3× per week) or the antimetastatic drug COL-3 (40 mg/kg daily) can result in effective tumor control. This improved efficacy of cytotoxic drugs with concomitant COX-2 suppression was correlated with increased apoptosis and decreased MVD. Because the COX-2–depleted tumor explants showed decreased MVD and increased apoptosis, the results corroborate the hypothesis that tumor-derived COX-2 plays a major role in tumor angiogenesis and growth, underlining the significance of COX-2 activity in tumor growth and its cytoprotective function in chemotherapy response.

A significantly decreased MVD count in the 6C+Dc group was further decreased after COL-3 or Taxotere treatment. These observations also corroborated well with the observed decrease in VEGF protein expression in 6C+Dc, in both the untreated and Taxotere-treated groups, as compared with the 6C−Dc untreated groups or even the 6C−Dc + Taxotere-treated groups (Fig. *6*). In light of this observation, it is conceivable that COX-2 suppression followed by significantly low level dosing with Taxotere (3× per week), often referred to as "metronomic dosing" (45), caused an antiangiogenic effect in the 6C+Dc group. These results are in agreement with the hypothesis that multiple exposures to low doses of chemodrugs act as an antiangiogenic agent (46). In this study, this antiangiogenic effect

was pronounced in the Taxotere-treated COX-2 suppressed group.

In summary, we have provided direct evidence to establish that constitutive induction of COX-2 is responsible for the increased resistance to chemotherapeutic drugs. Furthermore, inhibition of COX-2 expression, using an inducible COX-2 antisense cDNA construct, may abrogate this resistance. We propose an alternative approach composed of antisense COX-2 gene silencing and administration of significantly lower doses of chemotherapy to increase the efficacy of antitumor drugs against prostate cancer. Such an approach may further contribute to reducing the adverse side effects due to high-dose chemotherapy.

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